Conference Paper

# Anomalies in Natural Populations of Amphibians: Methodology for Laboratory Studies

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## Abstract

Recommendations are offered for the laboratory study of anomalies found in natural populations of amphibians to elucidate their causes. Various methods are mentioned, particularly breeding experiments, experimental gynogenesis and regeneration experiments.

**Keywords:** anomalies, methodology, amphibians, laboratory studies.

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Received: 23 January 2018
Accepted: 20 April 2018
Published: 3 May 2018

Publishing services provided by Knowledge E



Selection and Peer-review under the responsibility of the Amphibian and Reptiles Anomalies and Pathology Conference Committee.



## 1. Introduction

The study of anomalies in natural populations of amphibians can be very informative not only about these populations but also about exceptional aggressions, often of anthropic cause, suffered by ecosystems. For this reason, attention should be called to the presence and prevalence of anomalies in these natural populations. However such studies cannot be done only in the field. Observations made in the field can bring useful information but must be complemented by observations and experiments made in the laboratory.

## 2. The distinction soma-germen and its bearing on the study of anomalies

The distinction between germinal and somatic cells is crucial for understanding the causes of anomalies. Germinal cells are the ova and spermatozoa, and their precursor stages (oogonia, oocytes, ootids; spermatogonia, spermatocytes, spermatids): their DNA is transmitted to offspring. In contrast, the DNA of somatic cells (all other cells) is not transmitted. During the development of an organism, somatic cells may exert an epigenetic influence on the functioning of germinal cells and their DNA, but this

information is not passed to offspring. Therefore, although it is possible to categorise anomalies under many detailed headings, the most important difference is by far between genotypic anomalies (transmitted to offspring) and phenotypic anomalies (not transmitted to offspring).

Genotypic anomalies are determined by (genetic or chromosomal) mutations, which in their turn may have various causes (physico-chemical factors, 'spontaneous' mutations). Anomalies observed in some cases in hybrids also have genetic causes, being often due to perturbation of genetic regulation in composite organisms, but they are rarely transmitted to the offspring simply because hybrids are often sterile.

Phenotypic anomalies may have a much wider range of causes. These include accidents during development (traumatisms), physico-chemical aggressions (e.g. by pesticides or radiations), biological aggressions (e.g., by predation, parasitism, diseases, hormones, viruses) and, much more rarely, composite organisms like chimeras (resulting from partial fusion of eggs).

Of particular relevance to the study of the impact of human activities on the environment are all the anomalies caused by DNA damage due to major physico-chemical aggressions (mostly by radiation and chemicals). Two main categories of mutations must be distinguished here. Genic or chromosomal mutations induced in the DNA of somatic cells, which will not be transmitted to offspring, may be termed 'somatomutagenetic effects', whereas the term 'germinomutagenetic effects' may be used to designate genic or chromosomal mutations induced in DNA of germinal cells and liable to be transmitted to offspring. Somatomutagenetic effects include pathogenetic and carcinogenetic effects (physiological dysfunctioning, diseases, death) and teratogenetic effects (perturbation of growth and ontogenesis resulting in phenotypic anomalies). Germinomutagenetic effects include lethal mutations, sterility, aneuploidy and various phenotypic anomalies

## 3. The sources of variation in natural populations

Every natural population shows a range of variation in most morphological, anatomical and other characters. This variability has a double origin, genetic and epigenetic. For 'quantitative' characters showing a continuous variability, the distribution of this variation usually follows a normal distribution, with most specimens being close to a modal value for many characters. For 'qualitative' characters showing a discontinuous variability, with discrete character states, one often observes a polymorphism, i.e., the

presence within the population of different phenotypes in various proportions. Regarding so-called 'anomalies', the question is then: when should we speak of polymorphism and when should we speak of a rare mutation, possibly caused by an exceptional cause?

There is no clear-cut and 'scientific' reply to this question, as this is often just a matter of *a priori* convention. The most frequent convention states that an allele present at a frequency below 1% in a population is not considered as belonging to the natural polymorphism of this population but as an exceptional anomaly or mutation, as such a rate would correspond to spontaneous mutations likely to occur in each generation. But concretely, in studies of natural populations, the samples studied are often too low (below 100 specimens) to allow us to observe such rates, so in many cases the convention has moved up to 5%.

## 4. Main categories of anomalies

Although anomalies can potentially affect all aspects of the phenotype of organisms, as most of the surveys of anomalies in natural populations are carried out through study of specimens in the field, the great bulk of observations concern phenotypic anomalies that can be detected by macroscopic external inspection of specimens. In such conditions, the most frequently detected anomalies belong to the following categories:

### 4.1. Developmental anomalies

They include neoteny, paedogenesis, dwarfism, gigantism, etc. They result from phenomena of heterochrony (dissociation of development of different parts of the phenotype) or more generally of aneuchrony (acceleration of retardation, total or partial, of development).

### 4.2. Pigmentary anomalies

They include albinism, melanism, flavism, etc. They result from the absence of pigmentary cells or of the pigments themselves in these cells (e.g., melanin in albinism).

### 4.3. Limb and digit anomalies

They include polydactyly, ectrodactyly, syndactyly, polymely, etc. They result from perturbations in the development and growth or in the regeneration of the limb.

## 5. Anomalies as warning signals of environmental aggression or disruption

For a long time the study of anomalies in natural populations of amphibians has elicited little interest from institutional biologists, who considered it as 'anecdotal', especially as observations of anomalies were often isolated and as the causes of the anomalies often proved difficult to establish. Developmental biologists showed more interest in the production of anomalies in the laboratory (experimental teratology), which could shed light on the processes of development, differentiation and growth. But the growing interest in recent decades in the impact of human activity on ecosystems and their organisms has drawn attention to the study of anomalies in amphibian populations as potential warning signals of pollution or other kinds of environmental aggressions or disruptions (by radiation, chemicals, biological molecules, etc.). As a matter of fact, with their complex life cycle in different habitats, their direct contact with water, soil and air at all stages of their development and their large populations directly accessible to study, amphibians constitute an excellent group for the study of these questions.

## 6. The search for the causes of anomalies

Six main approaches may be used to try and clarify the causes of the anomalies: (1) phenotypic study; (2) breeding experiments (crossings); (3) gynogenesis; (4) regeneration experiments; (5) study of development and growth; and (6) survey of potential external factors.

### 6.1. Phenotypic study

The careful study of abnormal phenotypes and their comparison with normal phenotypes may be rich in information. For example, an abnormal phenotype may be 'harmonious' or not: an 'harmonious' phenotype points to it being the result of a spontaneous development, not of accidents, injuries, etc. The same is true of the

bilaterality and symmetry of the anomaly (for paired structures), and for gradients of severity (e.g., postero-anterior).

Information can also be derived from the association of anomalies in individuals or in populations. Some syndromes having identical or similar causes, like the anomaly P of *Pelophylax* frogs or some limb anomalies caused by mutations (see fig. 1), show a variability of severity in their expression and/or gradients of severity. They may be identified through the presence of diagnostic combinations of different anomalies having peculiar morphological characteristics. But not all associations of anomalies constitute syndromes. The presence of several distinct anomalies having clearly distinct causes (e.g., concerning the limbs and the coloration), in some individuals or in some populations of the same or different species, should particularly raise attention, as they may indicate the presence in the environment of strong mutagenetic or teratogenetic factors such as chemicals or ionising radiations.

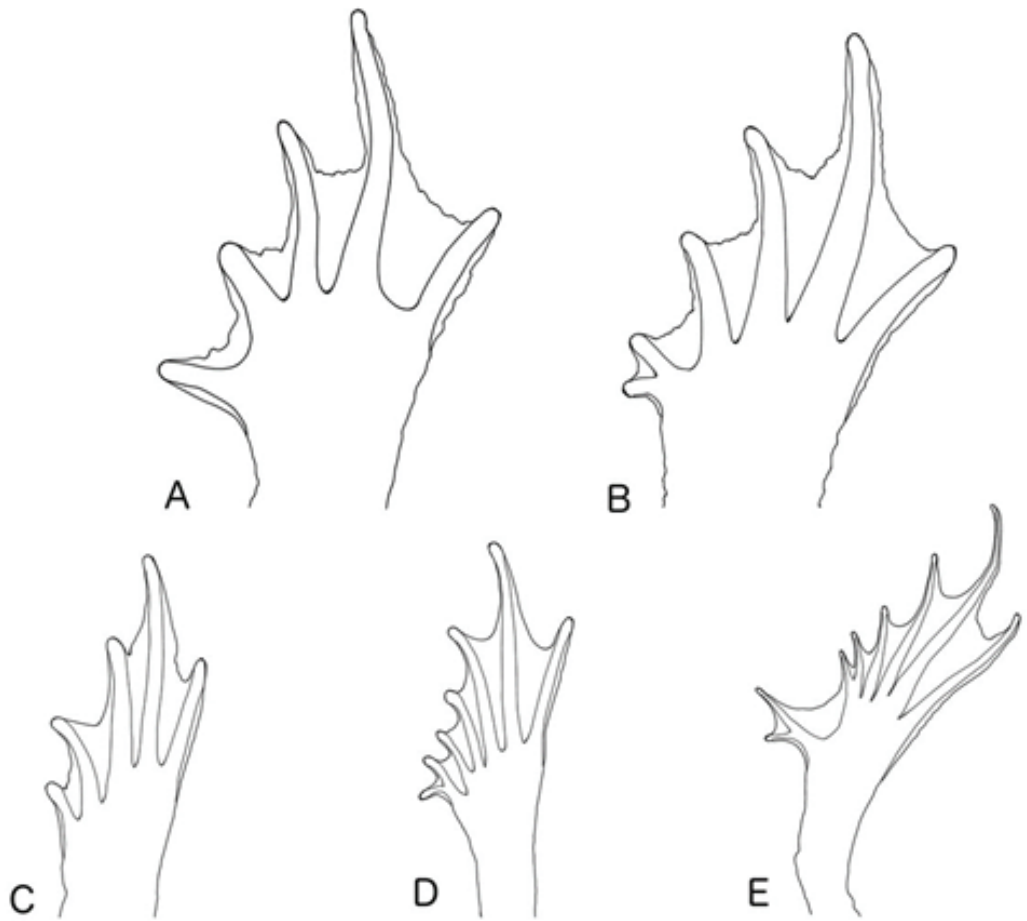


**Figure** 1: Some characteristic phenotypes of amphibian feet: (A) normal foot of *Bufo bufo*; (B) 6-toes polydactyly (6 toes) of genetic determinism in *Bufo bufo* (supernumerary toes shorter than first toe); (C) normal foot of *Pelophylax sp.*; (D) 7-toes polydactyly due to anomaly P in *Pelophylax sp.*; (E) 8-toes polydactyly due to anomaly P in *Pelophylax sp.* (internal supernumerary toes longer than first toe).

However, great caution is necessary in phenotypic study to avoid misleading assumptions.

First, one should beware of *phenocopies*. Developmental pathways of organisms are quite constrained and cannot take any direction, so very similar phenotypes can result from very different causes. For example, polydactyly (additional digits) may result from dominant or recessive alleles or from non-genetic causes (e.g., anomaly P, hyperregeneration). Albinism, the absence of pigment, may result from mutations affecting the chain of pigment synthesis at different stages, from the absence of pigmentary cells or from hormonal causes (leading to pigment contraction around cell nuclei).

Statistics should also be treated with caution, as probabilities are not causes and may be misleading. More generally, a correlation is not a cause. Two phenomena that are correlated may be due to a third, unknown or unidentified one. Phenotypic studies often suggest possible causes for anomalies, but then the latter should be demonstrated.

## 6.2. Breeding experiments

Crossings involving abnormal specimens may be very informative. Several kinds of crossings may be carried out.

(1) A crossing between an abnormal specimen $S_{Aa}$ from population A and a normal specimen $S_{Bn}$ from remote population B may suggest the existence in S of a dominant allele responsible for the anomaly observed (50% of the offspring will then exhibit the anomaly), but the absence of abnormal specimens in the offspring is inconclusive.

(2) A crossing between an abnormal specimen $S_{Aa}$ and a normal specimen $S_{An}$ from the same population A may suggest the existence in $S_{Aa}$ of either a dominant mutant allele or a recessive allele also present but 'hidden' in $S_{An}$ (50% of abnormal offspring in both cases), but the absence of abnormal specimens in the offspring is inconclusive.

(3) A crossing between two specimens $S_{A1}$ and $S_{A2}$ from population A showing similar anomalies may be informative in case of dominant mutation (75% of the offspring affected, or 67% if homozygosity for this allele is lethal) or of recessive mutation (100% affected), whereas the absence of abnormal specimens in the offspring suggests a non-genetic cause.

(4) In all the previous cases, definitive conclusions will require backcrosses between themselves or with their parents of the F1 specimens obtained from the initial crosses.

Breeding experiments can often be done easily with specimens just collected during the breeding season: it is often sufficient to place a pair in water in captivity to recover fertilised eggs the next morning. But this unsophisticated method has a drawback: it does not allow us to make control crosses.

As a matter of fact, for clear results which avoid misinterpretations, it is highly advisable to care for making control crosses. For this, recourse to artificial fertilisation is useful, as it allows us to fertilise the ova of several females by a single male and vice versa. A female can be induced to ovulate by hormonal injections, and spermatozoa from a male can be recovered by crushing its testicles in distilled water or in physiological saline solution. If possible, it is better to use control specimens from different populations: controls from the same population as the abnormal specimen always carry the risk of using a seemingly 'normal' specimen heterozygous for a recessive allele responsible for the anomaly whenever in a homozygous condition.

Other precautions should be taken in the interpretation of the results of crosses. Here also, the possibility of phenocopies should be considered. For example, when two albino specimens are crossed, it is not unusual to recover 100% normally pigmented specimens in the offspring. This may be due to albinism in the two parents being caused by non-homologous mutations affecting pigmentation, for example for cutting the chain of synthesis of the pigment at different stages. In such cases, the absence of albinotic specimens in the F1 should not be interpreted as meaning that albinism in the parents was not due to genetic causes. In such cases, F1 specimens should be crossed among themselves or backcrossed with the parents, which allows us to recover albino specimens homozygous for one or both recessive alleles. Interpretation of crosses should beware of variability and incomplete genic expression (variability in penetrance), often linked to epigenetic influences (see fig. 2).

## 6.3. Experimental parthenogenesis and gynogenesis

Natural parthenogenesis exists in nature in various zoological groups. Experimental parthenogenesis has been obtained by biologists through various techniques since the end of the $19^{th}$ century. Most of these techniques (e.g., pricking frog eggs with a needle) are quite hard to implement and have a very low rate of success. But a very efficient technique is that of experimental gynogenesis. It can be realised by different

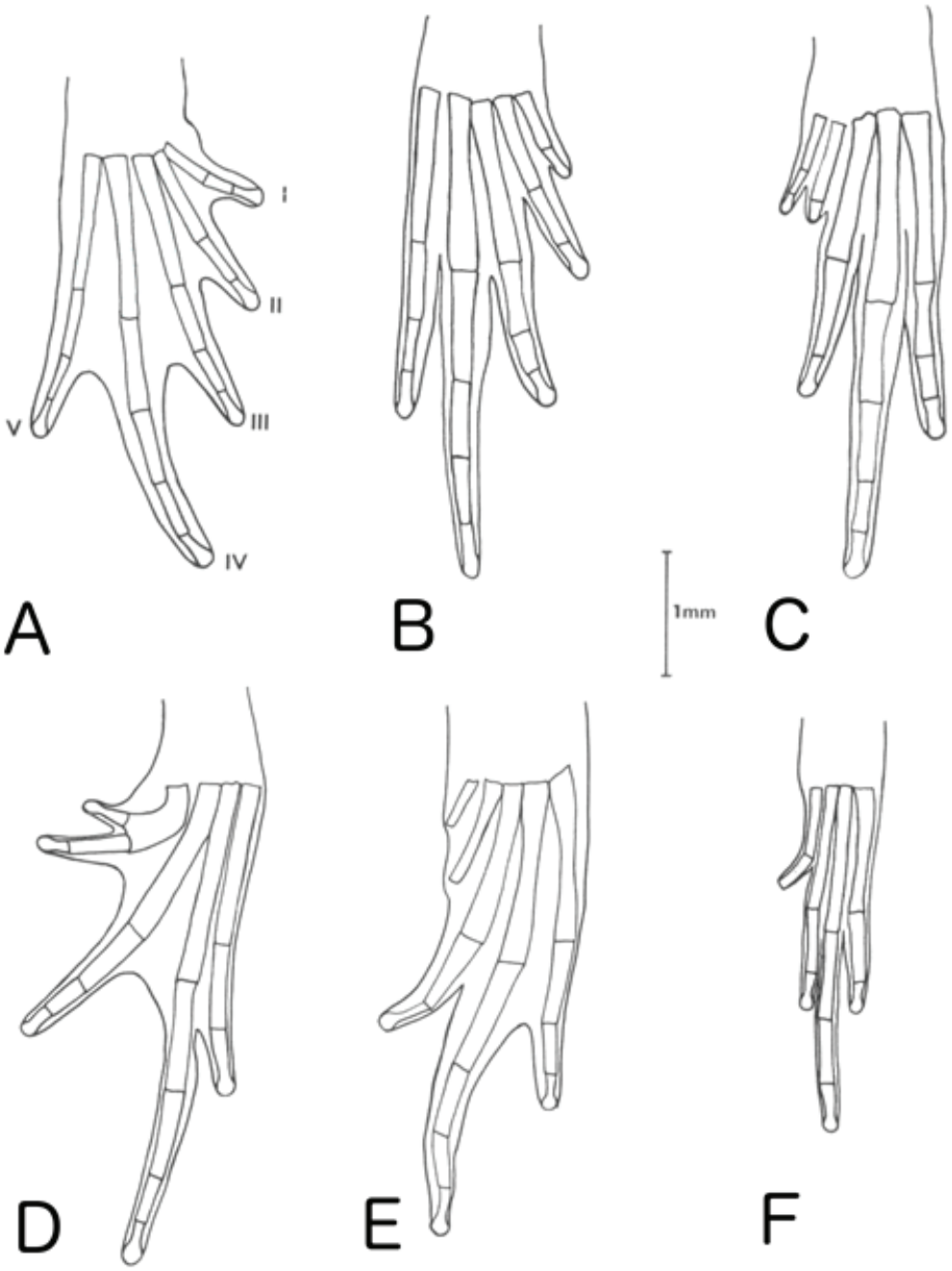


**Figure** 2: Variability of the No syndrome of ectrodactyly and hypophalangy in *Rana temporaria*.

methods. One of them consists in irradiating some sperm (in order to destroy the nuclei of the spermatozoa) and putting it in contact with haploid ova. In such conditions, the spermatozoa may stimulate the development of ova without introducing genetic material into them. The development of such haploid ova usually does not go very far. But their chromosomal complement can be doubled just after their stimulation, for example by heat shock or hydrostatic pressure. Such diploid ova are then homozygous for all their alleles (except for mutations occurring after their diploidisation) and they

can then undergo a complete development (except when they carry recessive alleles that are lethal in a homozygous condition).

This technique of experimental gynogenesis is very efficient for the exploration of the gene pools of individuals or even populations. Table 1 compares the respective efficiency of inter-individual crosses and gynogenesis to establish whether an anomaly is determined by a dominant or recessive allele of a non-genetic factor. Whereas two generations are necessary to establish this by crosses, gynogenesis allows us to do it in one generation. Of course, these approaches are not sufficient to understand the causes of all anomalies: those which have a polygenic determinism, are linked to sex or are determined by mitochondrial genes (or by a combination of genetic and epigenetic factors) require more study, but at least these approaches allow to clarify the simple, monofactorial cases.

Table 1: Comparison of the efficiency of inter-individual crosses and experimental gynogenesis to distinguish three simple determinisms of anomalies (dominant allele, recessive allele, non-genetic).

| | Crossing | | Gynogenesis |
|---|---|---|---|
| | **F1: A × N** | **F2: A × A** | **A** |
| **Genetic: dominant allele** | 50% A | 75% A | 50% A |
| | 50% N | 25% N | 50% N |
| **Genetic: recessive allele** | 100% N | 25% A | 100% A |
| | | 75% N | |
| **Non-genetic** | 100% N | 100% N | 100% N |

(N: normal phenotype; A: abnormal phenotype.)

So far, the gynogenetic approach had been used in studies of amphibian anomalies only to elucidate the determinism of anomalies observed in amphibian females. But this approach could also be used much more widely in order to evaluate the 'mutation loads' of amphibian populations, through applying it to hundreds of eggs from many different individuals. This could allow us to compare this load between populations living in different environments and to formulate hypotheses regarding the respective 'mutagenicity' of these populations or of their environment.

## 6.4. Regeneration experiments

In all larval anuran and urodelan amphibians, as well as in adult urodelans and a few adult anurans, the amputation of a limb, of digits, of tail or of a few other parts of body (e.g. eyes) is normally followed by the regeneration of this structure (fig. 3). The artificial amputation of an abnormal organ (e.g., a polydactylous limb) may bring

useful information on the cause of the anomaly (fig. 4): if the regenerated structure still shows the anomaly, this indicates that the factor responsible for it is still active in the individual (which is compatible with a genetic cause) but if the anomaly is not present in the regenerated organ, this suggests that this factor has disappeared and therefore that was not of a genetic but epigenetic nature.

For such experiments, attention should be given to the developmental stage at which the amputation is effected (in most anurans there is no regeneration after metamorphosis), and hyperregeneration must be considered as a possible cause for anomalies (especially when these are not 'harmonious' in aspect). Anyway, in such experiments like crosses, it is always important to care about having controls

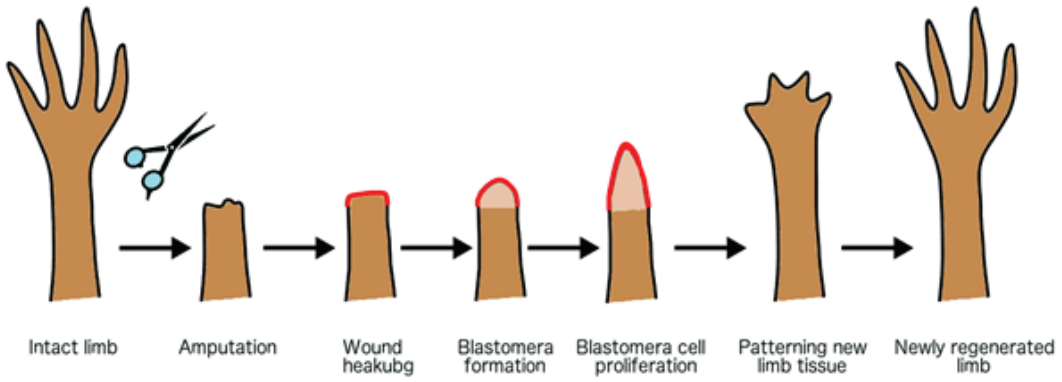


**Figure** 3: Schematic representation of regeneration following amputation of hand in an amphibian.

## 6.5. Study of development and growth

Some anomalies may change during development and growth. They may have a delayed appearance or, on the contrary, disappear after some time, or they may go through different subsequent stages. The latter case is particularly frequent with anomalies of coloration: in some cases, the skin may be transparent in early stages and become pigmented after some time (weeks, months or years), or the distribution of zones of abnormal coloration on body may change. This may occur both with genetic anomalies (such as black eyes and associated pigmentary anomalies) and in non-genetic ones (caused by hormonal disruptions, parasites, viruses, etc.). For this reason, abnormal specimens found in the field or obtained from eggs in the laboratory should preferably be kept alive in captivity for years. Beside the fact that this will allow us to use them in crossing or gynogenesis experiments, this is by itself much more informative than just photographing them once and releasing them, or killing and fixing them for morpho-anatomical study.

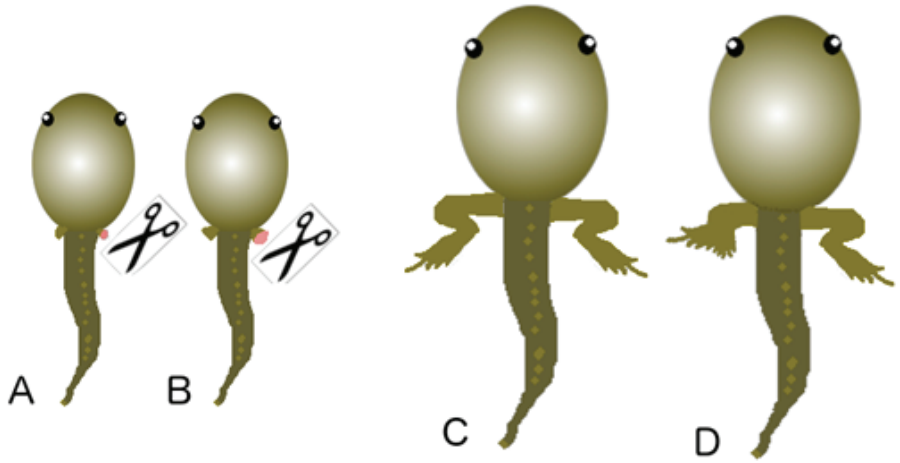


**Figure** 4: Schematic representation of ablation of the right hind limb bud in a normal tadpole of *Pelophylax* (A, C) and in one affected by anomaly P (B, D). Note that in D the right hind limb regenerated as normal whereas the left one showed polydactyly.

### 6.6. Survey of potential external factors

As discussed in the companion paper by Ohler & Dubois in this volume, it may be useful to carry out a survey of potential external factors in the environment that might act as causes for phenotypic anomalies (e.g., chemicals, fertilisers, hormones, viruses, diseases, parasites, predators).

Care should however be exerted to avoid the major pitfalls of such approaches. The most important (and often overlooked) one is that *a correlation is not a cause*. The presence of a potentially mutagenic, teratogenic or pathogenic factor in a habitat is not sufficient evidence *by itself* that it is responsible for an anomaly observed in the population. A causal relationship between the two observed facts must be demonstrated.

## 7. Laboratory equipment and facilities

Some laboratory equipment and facilities are indispensable for carrying out efficiently serious scientific studies on anomalies in amphibian populations. They include breeding facilities for amphibians (from egg to adult) in which the main environmental

conditions (e.g., temperature and light) are controlled, breeding facilities for prey and devoted technical staff.

Equipment is needed for spontaneous crosses, artificial fertilisation and gynogenesis (e.g., for the irradiation of sperm and for heatshock and hydrostatic pressure): this includes a cytogenetic facility (to check ploidy) and good quality optical equipment. In order to have these facilities and equipment at hand whenever abnormal specimens are collected in the field or obtained in captivity, a laboratory should be conceived, prepared and equipped. Serious study in this poorly known and complex field cannot be improvised suddenly when 'strange' specimens are found, or the conclusions drawn from their study may be fragile or misleading.

## References

A list of references on these questions would occupy many pages and cannot be provided here. Such references are available in the volume on anomalies in natural populations of amphibians edited by K. Henle, A. Dubois and V. Vershinin and published in *Mertensiella*.